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Modulation of M_2 muscarinic receptor–receptor interaction by immunoglobulin G antibodies from Chagas' disease patients

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Summary

Circulating immunoglobulin (Ig)G antibodies against M_2 muscarinic acetylcholine receptors (M_2 mAChR) have been implicated in Chagas' disease (ChD) pathophysiology. These antibodies bind to and activate their target receptor, displaying agonist-like activity through an unclear mechanism. This study tested the ability of serum anti-M_2 mAChR antibodies from chronic ChD patients to modulate M_2 muscarinic receptor–receptor interaction by bioluminescence resonance energy transfer (BRET). Human embryonic kidney (HEK) 293 cells co-expressing fusion proteins M_2 mAChR-Renilla luciferase (RLuc) and M_2 mAChR-yellow fluorescent protein (YFP) were exposed to the serum IgG fraction from ChD patients, and BRET between RLuc and YFP was assessed by luminometry. Unlike serum IgG from healthy subjects and conventional muscarinic ligands, ChD IgG promoted a time- and concentration-dependent increase in the BRET signal. This effect neither required cellular integrity nor occurred as a consequence of receptor activation. Enhancement of M_2 receptor–receptor interaction by ChD IgG was receptor subtype-specific and mediated by the recognition of the second extracellular loop of the M_2 mAChR. The monovalent Fab fragment derived from ChD IgG was unable to reproduce the effect of the native immunoglobulin. However, addition of ChD Fab in the presence of anti-human Fab IgG restored BRET-enhancing activity. These data suggest that the modulatory effect of ChD IgG on M_2 receptor–receptor interaction results from receptor cross-linking by bivalent antibodies.

Keywords: BRET, Chagas, IgG, M_2 mAChR, receptor–receptor interaction

Introduction

Circulating immunoglobulin (Ig) G antibodies against M_2 muscarinic acetylcholine receptors (M_2 mAChR) have been implicated in Chagas' disease (ChD) pathophysiology [1–5]. These antibodies are highly prevalent in chronic ChD patients with sinus node dysfunction [6], achalasia [4] and megacolon [5]. Because there is a strong association between anti-M_2 mAChR antibodies and dysautonomia [1,2], these antibodies have been proposed as a marker for cardiac autonomic dysfunction in chronic ChD patients [3]. Serum anti-M_2 mAChR antibodies with clinical significance have also been described in patients suffering from idiopathic dilated cardiomyopathy [7] and hypertrophic cardiomyopathy [8].

Although the precise mechanism of action of ChD anti-M_2 mAChR antibodies is still unclear, previous reports agree that these antibodies recognize the second extracellular loop (II-ECL) of M_2 mAChR [2,9], and subsequently activate their target receptor, displaying agonist-like activity [1,10,11]. In fact, anti-M_2 mAChR antibodies decrease cyclic adenosine-5′-monophosphate (cAMP) accumulation [10,11], enhance cyclic guanosine monophosphate (cGMP) production [10,11] and inhibit L-type Ca^{2+} currents [12]. As a result, they elicit negative inotropic and chronotropic effects in the rodent myocardium [1–3,6,9,10] and increase tonic contraction of rat oesophageal and colonic smooth muscle [4,5], mimicking the effects of muscarinic partial agonists. Radioligand binding studies have shown that these antibodies also inhibit the specific binding of classical muscarinic antagonists to the M_2 receptor in cardiac and gastrointestinal smooth muscle membrane preparations [1,5,10–13]. Because the serum IgG fraction from ChD patients can increase the efficacy and affinity of agonists and decrease the affinity of antagonists on cardiac muscarinic

receptors, anti-M_2 mAChR antibodies are also believed to act as muscarinic allosteric modulators [13].

Classical pharmacological and biochemical studies as well as modern biophysical approaches based on bioluminescence or fluorescence resonance energy transfer [e.g. bioluminescence resonance energy transfer (BRET) or fluorescence resonance energy transfer (FRET)] have provided evidence to support that mAChR form constitutive dimers or higher-order oligomers [14–19]. In particular, the M_2 receptor subtype has been shown to form homotropic complexes (dimers or tetramers) [17,19] and heterodimers with either other mAChR subtypes [15,17] or non-muscarinic G-protein coupled receptors [18]. Receptor–receptor interactions involving M_2 mAChR have been implicated in essential aspects of receptor pharmacology, such as ligand binding [15], signalling [20], long-term regulation [17] and trafficking [18].

Given the pharmacological relevance of muscarinic receptor oligomerization and the ability of serum antibodies from ChD patients to bind to and activate M_2 mAChR, we assessed whether these anti-autonomic receptor antibodies would modulate M_2 muscarinic receptor–receptor interactions. The present study shows that circulating IgG antibodies against M_2 mAChR from chronic ChD patients enhance M_2 mAChR receptor–receptor interaction by receptor cross-linking. In addition, it analyses the specificity of antibody–receptor interaction at the molecular level, and discusses the implications of these findings in ChD pathophysiology.

Materials and methods

Human sera

Serum samples were obtained from 15 patients with chronic ChD and 15 control healthy subjects recruited at the D. F. Santojanni Hospital, Buenos Aires, Argentina. Diagnosis of ChD was made on the basis of three standard serological reactions against *Trypanosoma cruzi*: indirect haemagglutination, indirect immunofluorescence and enzyme linked-immunosorbent assay (ELISA). ChD patients showed signs and symptoms of chronic heart disease – with or without congestive heart failure – and autonomic nervous system dysfunction (dysautonomia), as shown by abnormal responses to two or more of the following diagnostic tests: Valsalva manoeuvre, tilting, hyperventilation and coughing tests [1,2]. Control subjects exhibited negative serology for *T. cruzi* infection and normal tests for dysautonomia. All methodologies used in this study conformed to the standards set by the Declaration of Helsinki. Every ChD patient or healthy subject gave fully informed consent under a protocol approved by the Santojanni Hospital's Ethics Committee. The presence of anti-M_2 mAChR in ChD patients was detected using the immunoenzymatic protocol described below. Although all ChD sera tested positive, only those yielding optical density readings at 405 nm (OD_{405nm}) between 1·0 and 1·5 were selected for IgG purification and further studies.

Purification of serum IgG and Fab fragments

Serum IgG fractions from selected ChD patients and control subjects were purified by diethylaminoethyl (DEAE) cellulose chromatography, as described previously [10]. Fab fragments from ChD or control IgG were prepared using immobilized papain (Pierce, Thermo Fisher Scientific, Rockford, IL, USA) followed by chromatography through protein G-agarose (Roche Diagnostics, Indianapolis, IN, USA) to remove undigested IgG and Fc fragments. Protein concentration of IgG and Fab samples were determined by Lowry's method [21].

Purification of monospecific anti-M_2 mAChR antibodies

ChD IgG fractions were subjected to affinity chromatography against a synthesized 25-mer-peptide (pM_2: VRTVEDGECYIQFFSNAAVTFGTAI), corresponding to the amino acid sequence of the II-ECL of human M_2 mAChR (residues 169–193) as described previously [2]. The non-anti-pM_2 fraction was first eluted with PBS and specific anti-pM_2 antibodies were then eluted with 3 M KSCN, 1 M NaCl. Both IgG fractions were dialysed against phosphate-buffered saline (PBS) and concentrated by ultrafiltration. Immune reactivities of non-anti-peptide and monospecific anti-pM_2 IgG fractions were monitored by ELISA.

ELISA

The pM_2 peptide was coated onto microtitre plates (2·5 μg/well) in 0·1 M Na_2CO_3 buffer pH 11, for 18 h at 4°C. The wells were then saturated with 10% v/v fetal bovine serum (FBS) in PBS (FBS/PBS) for 2 h at 37°C. One hundred microlitres of dilutions of patient sera (1:50) or purified IgG fractions in PBS/bovine serum albumin (BSA) 1% were allowed to react with the peptide for 2 h at 37°C. After the wells were washed three times with 0·05% Tween-20 in PBS, 100 μl of alkaline phosphatase-conjugated goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:6000 in PBS/BSA 1% was added to each well and allowed to bind for 1 h at 37°C. OD_{405nm} was measured after the incubation with 1 mg/ml p-nitrophenyl phosphate for 30 min at room temperature. Sera with OD values higher than the mean + 3 standard deviations (s.d.) of control sera were taken as positive for anti-pM_2 antibodies. The presence of anti-pM_2 antibodies in ChD IgG fractions after affinity chromatography was confirmed by performing ELISA inhibition tests. These experiments were carried out by pre-incubating various amounts of anti-pM_2 IgG or non-anti-pM_2 IgG fractions in the presence or absence of soluble peptide (pM_2) for 60 min at 37°C, before incubating either IgG fraction with the immobilized peptide.

Plasmids, cell culture and transfection

M_2 and M_3 mAChR-RLuc and mAChR-YFP fusion protein constructs were generated by ligating humanized Renilla luciferase (RLuc) or enhanced yellow fluorescence protein (YFP) moieties to the C-terminal end of the respective receptors. Details regarding the construction procedures as well as the pharmacological characterization of all fusion proteins have been described in a previous study [17]. Human embryonic kidney (HEK) 293 cells were grown in Dulbecco's modified Eagle's medium (Applichem GmbH, Darmstadt, Germany) supplemented with 10% FBS, penicillin (100 units/ml) and streptomycin sulphate (0·1 mg/ml) at 37°C in a humidified 5% CO_2 environment. Transient transfections were performed on 70–80% confluent cells using a calcium phosphate precipitation protocol [22].

BRET assays on cells

To monitor receptor–receptor interactions in living cells, BRET assays were performed on cells co-expressing M_2-RLuc (or M_3-RLuc) and M_2-YFP (or M_3-YFP) at equimolar amounts. Expression levels of donor and acceptor fusion proteins were determined, respectively, by measuring luminescence and fluorescence in each BRET experiment and correlating those values with the amount of mAChR-binding sites in the same cells, as described previously [17] (data not shown). Thirty-six h after transfection, cells were washed with warm PBS and resuspended in modified Krebs–Ringer–HEPES buffer (104 mM NaCl, 5 mM KCl, 1·2 mM $MgSO_4$, 1·2 mM $CaCl_2$, 1·2 mM Na_2HPO_4, 10 mM glucose, 20 mM HEPES, pH 7·4) supplemented with 0·1% BSA (KRHA) [23]. Transfected cells were then placed in 1·5 ml-clear microcentrifuge tubes at a density of 100 000 cells/tube, and treated with different concentrations of muscarinic ligands or antibodies (IgGs or Fab fragments) at room temperature for the desired time, from 15 to 60 min. Coelenterazine h (Promega, Madison, WI, USA) was added at a final concentration of 5 μM, and readings were collected 15 min later in a Turner 20/20 luminometer (Turner Designs, Sunnyvale, CA, USA), which allowed the sequential integration of the emission signals to be detected in the 510–590 nm and 440–500 nm windows by using filters with the appropriate band pass [24]. The BRET signal was defined as the ratio of the light intensity measured at 510–590 nm over 440–500 nm. BRET signal values were transformed into BRET ratio values by subtracting the background signal detected when the receptor-RLuc constructs were expressed alone. Consequently, the BRET ratio was defined as [(emission at 510–590 nm) – (emission at 440–500 nm) × Cf]/(emission at 440–500 nm), where Cf refers to (emission at 510–590 nm)/(emission at 440–500 nm) for the corresponding mAChR-RLuc expressed alone [25]. Because the BRET ratio may vary according to the presence of IgG fractions (or their Fab fragments) from different species, the effects of human IgGs or Fabs were normalized by that of a standard IgG (normal goat IgG) (or its Fab fragment), at the same concentration. Goat IgG was chosen because its effect on BRET was similar to the average effect of human IgG from control subjects. Consequently, net changes in BRET ratio values obtained following treatment with muscarinic ligands, human IgGs or their Fab fragments, were expressed as ΔBRET = (BRET ratio after treatment with muscarinic ligands, human IgGs or Fab fragments)–(BRET ratio after treatment with buffer alone, goat IgG or Fab from goat IgG, respectively). This correction also allowed us to compare ΔBRET values from different experiments. All BRET ratio and ΔBRET values were multiplied by 1000 and expressed as milibrets (mB).

BRET assays on membranes

Membranes were prepared from cells expressing M_2-RLuc alone or co-expressing M_2-RLuc and M_2-YFP at equimolar numbers using the transfection protocol described above. Transfected cells were washed twice with warm PBS and then glass–glass homogenized ×20 in ice-cold buffer containing 50 mM Na/KPO_4, pH 7·4, 1 mM ethylenediamine tetraacetic acid (EDTA) and a protease inhibitor cocktail [26]. Homogenates were centrifuged at 1000 ***g*** for 5 min at 4°C to remove nuclei and unbroken cells. The supernatant was then centrifuged at 10 000 ***g*** for 20 min, and the pellet was washed twice in the same buffer. Membrane preparations were stored at −80°C until used. For BRET assays, membranes were diluted 1:10 with KRHA buffer or 5 mM Na/KPO_4, pH 7·4 supplemented with BSA 0·1%. The remaining part of the procedure was the same as that used for cells.

Statistical analysis

Data were analysed using Prism 5 software (GraphPad, La Jolla, CA, USA). One- or two-way analyses of variance followed by Bonferroni *post-hoc* tests were used as required. Results were expressed as means ± standard error of the mean (s.e.m.). Differences between means were considered significant if $P < 0{\cdot}05$.

Results

Effect of ChD IgG on BRET in cells and membranes

HEK 293 cells were co-transfected with M_2-RLuc and M_2-YFP at a 1:3 DNA ratio, which allowed equimolar expression of both fusion proteins, according to a previous study [17]. Under these conditions the total amount of expressed M_2 mAChR binding sites ranged from 140 to 350 fmol/mg cell protein, as determined by a [^{3}H]-quinuclidinyl benzilate (QNB) binding assay. These values are consistent with physiological levels of receptor expression [17]. Addition of RLuc substrate coelenterazine h to co-transfected cells generated a

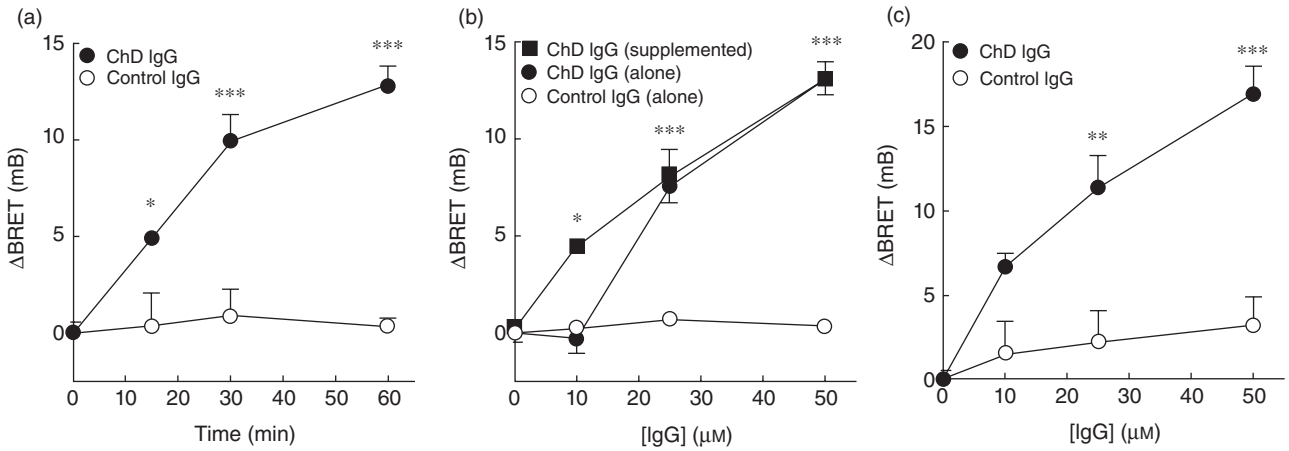

Fig. 1. Effect of Chagas' disease (ChD) immunoglobulin (Ig)G on M_2 muscarinic receptor–receptor interaction in cells and membranes. Human embryonic kidney (HEK) 293 cells were co-transfected with M_2 mAChR-Renilla luciferase (M_2-RLuc) and M_2 mAChR-yellow fluorescent protein (M_2-YFP). After 36 h, intact cells were treated with 50 μM ChD or control IgG for various incubation times (a) or different concentrations of either immunoglobulin for 60 min (b). Alternatively, cells were incubated for 60 min in the presence of different concentrations of ChD IgG and supplementary amounts of control IgG, keeping a total IgG concentration of 50 μM (b). Crude membranes obtained from HEK 293 cells co-expressing M_2-RLuc and M_2-YFP were treated with various concentrations of ChD or control IgG for 60 min (c). Net changes in bioluminescence resonance energy transfer (BRET) values (ΔBRET) are presented as mean ± standard error of the mean of five to seven different patients tested in three to five independent experiments. Significant differences *versus* control IgG for the same incubation time or IgG concentration (*$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$).

basal BRET ratio of 50–65 mB. Quantitative analysis of receptor–receptor interaction has demonstrated that this basal BRET value is not a consequence of receptor construct over-expression but the result of constitutive M_2 receptor–receptor interaction [17].

Treatment of co-transfected cells with ChD IgGs promoted a time- and concentration-dependent increase in the BRET ratio, while control IgGs induced a negligible effect which did not change over time (Fig. 1a,b). Because we noticed that ChD IgG (10 μM) was ineffective, we investigated the influence of total IgG concentration on the BRET signal. We exposed our M_2-RLuc/M_2-YFP BRET cell system to various concentrations of ChD IgG in the presence of additional amounts of control IgG, keeping a total IgG concentration of 50 μM. Under these conditions, ChD IgG (10 μM) led to a significant increase in the BRET ratio, suggesting that the presence of a carrier immunoglobulin fraction increases the specific interaction of BRET-enhancing antibodies with the M_2 mAChR BRET pair (Fig. 1b).

With regard to the specificity of the carrier protein in our BRET assay, we compared the carrier effect of control IgG with that of goat IgG or BSA. Briefly, treatment of our cellular M_2-RLuc/M_2-YFP BRET system with 10 μM ChD IgG supplemented with 40 μM goat IgG led to a significant increase in BRET, compared with 10 μM control IgG supplemented with 40 μM goat IgG or 10 μM ChD IgG alone (Supplementary Table S1). Under identical experimental conditions, the addition of supplementary amounts of BSA could not enhance the effect of 10 μM ChD IgG on BRET. These results indicate that goat IgG, but not BSA, is capable of mimicking the carrier effect of control IgG. Although these findings would suggest that the carrier effect is specific of IgG, other proteins with various physicochemical properties should be tested in order to define the specificity of the carrier protein.

Treatment of membranes from cells co-expressing M_2-RLuc and M_2-YFP with ChD IgG resulted in a similar concentration-dependent increase in the BRET signal, indicating that energy transfer between these fusion proteins does not require cellular integrity, and occurs at the cellular membrane level (Fig. 1c). The effect of 10 μM ChD IgG on BRET appears to be higher than that promoted by control IgG at the same concentration; however, both ΔBRET values are not statistically different ($P > 0{\cdot}05$). Therefore, we should not rule out the need of a carrier protein to obtain significant differences between ChD and control BRET values in membranes at low IgG concentrations.

Receptor-subtype specificity

ChD IgG was unable to modify basal BRET signals in cells expressing alternative BRET pairs, such as M_3-RLuc/M_3-YFP, M_2-RLuc/M_3-YFP or M_3-RLuc/M_2-YFP. Resulting BRET values were similar to those obtained in cells treated with IgG from control subjects (Fig. 2). These data suggest that the effect of ChD IgG on muscarinic receptor–receptor interaction results from the specific recognition of the M_2 muscarinic receptor subtype.

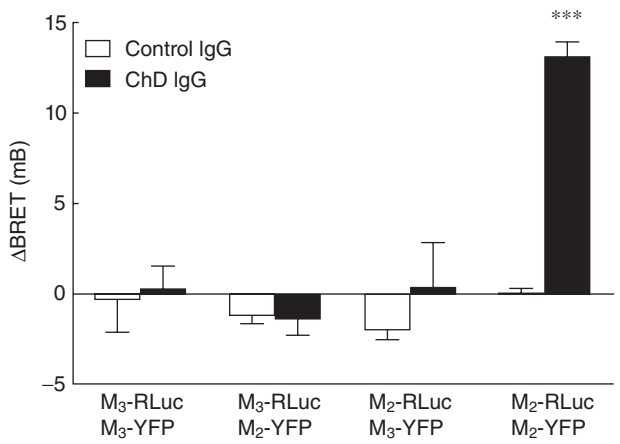

Fig. 2. Receptor subtype specificity of bioluminescence resonance energy transfer (BRET)-enhancing activity. Human embryonic kidney 293 cells co-expressing various BRET pairs (mAChR-Renilla luciferase/mAChR-yellow fluorescent protein: M_3-RLuc/M_3-YFP, M_3-RLuc/M_2-YFP, M_2-RLuc/M_3-YFP or M_2-RLuc/M_2-YFP) were treated with 50 μM Chagas' disease (ChD) or control immunoglobulin (Ig)G for 60 min. ΔBRET values are presented as mean ± standard error of the mean of five different patients tested in five to seven independent experiments. Significant differences *versus* other BRET pairs treated either with ChD or control IgG (***$P < 0{\cdot}001$).

Effects of conventional muscarinic ligands

The effect of ChD IgG on M_2 muscarinic receptor–receptor interaction was compared to that of conventional muscarinic ligands. Unlike ChD IgG, a full agonist (carbachol), a partial agonist (pilocarpine), an antagonist (atropine) and an allosteric modulator (gallamine) were unable to enhance the BRET signal in the cellular M_2-RLuc/M_2-YFP expression system, compared with buffer alone (KRHA) (Fig. 3). Moreover, a tendency for slightly decreased BRET values in the presence of all muscarinic ligands tested was observed, especially at the highest ligand concentrations. Slight variations in energy transfer promoted by 10 μM carbachol could not be antagonized by 10 μM atropine (Supplementary Fig. S1), suggesting that they do not result from receptor activation or any other event downstream. Rather, they seem to reflect negligible changes in constitutive M_2 receptor–receptor interaction, in agreement with our previous report [17].

Involvement of the II-ECL of the M_2 mAChR

Affinity chromatography of the whole IgG from ChD patients against an immobilized peptide corresponding in sequence with the II-ECL of human M_2 receptor (pM_2) resulted in the isolation of two antibody fractions: non-anti-pM_2 IgG, eluted with PBS, and anti-pM_2 IgG, eluted with 3 M KSCN, 1 M NaCl. Immune reactivity against pM_2 was tested on both fractions by ELISA inhibition tests, which confirmed the efficacy of the chromatographic procedure (Supplementary Fig. S2).

In terms of reactivity against pM_2, preliminary ELISA data had demonstrated that a 1–2 μM concentration of anti-pM_2 ChD IgG was equivalent to a 50 μM concentration of the total serum IgG fraction for a given ChD patient (data not shown). Therefore, we tested the BRET activity of anti-pM_2 fractions IgG (2 μM) from various ChD patients. For the purpose of the BRET assay, these fractions were supplemented with either control or non-anti-pM_2 IgG fractions as carrier immunoglobulins, keeping a total IgG concentration of 50 μM, according to the data discussed above (Fig. 1b). Under these conditions, the anti-pM_2 ChD IgG fraction promoted a significant increase in the BRET signal (Fig. 4a). As expected, the effect of 50 μM non-anti-pM_2 ChD IgG alone was similar to that of control IgG at the same concentration. These results were confirmed by performing concentration–response curves of anti-pM_2 ChD IgG supplemented with non-anti-pM_2 ChD or control IgG, as indicated above. As shown in Fig. 4b, monospecific anti-pM_2 antibodies promoted maximal increases in energy transfer of $16{\cdot}0 \pm 4{\cdot}2$ and $14{\cdot}1 \pm 3{\cdot}8$ mB, respectively, which were similar to the BRET ratio induced by 50 μM total ChD IgG ($13{\cdot}0 \pm 1{\cdot}32$ mB) (Fig. 4a).

Because both anti-M_2 mAChR antibodies and muscarinic allosteric ligands had been shown to interact to the acidic amino acid cluster (EDGE) at the II-ECL of M_2 receptor [27], we investigated whether a muscarinic allosteric modulator could modulate the BRET-enhancing activity of ChD anti-M_2 mAChR antibodies. Membranes from cells expressing the M_2Rluc/M_2-YFP BRET pair were incubated with 50 μM control IgG or ChD IgG in the presence or absence of

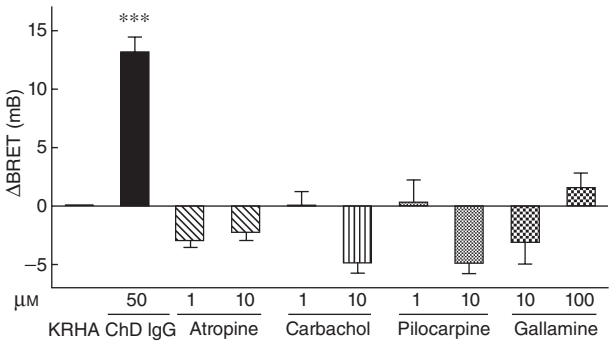

Fig. 3. Effects of conventional muscarinic ligands on M_2 muscarinic receptor–receptor interaction. Human embryonic kidney 293 cells co-expressing M_2 mAChR-Renilla luciferase and M_2 mAChR-yellow fluorescent protein were treated with carbachol, atropine, pilocarpine, gallamine or Krebs–Ringer–HEPES supplemented with 0.1% BSA (KRHA) for 60 min. Chagas' disease (ChD) immunoglobulin (Ig)G was also tested at equal conditions as our positive control. Δ-bioluminescence resonance energy transfer (ΔBRET) values are presented as mean ± standard error of the mean from four to six independent experiments performed in duplicate. Significant differences *versus* all muscarinic ligands (***$P < 0{\cdot}001$).

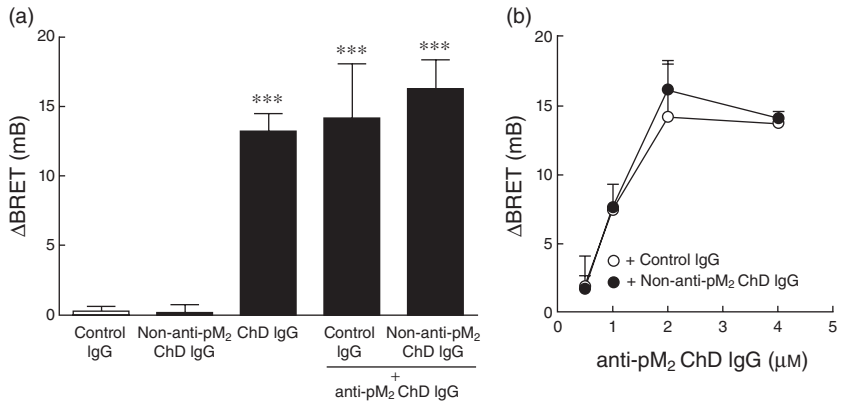

Fig. 4. Epitope specificity of bioluminescence resonance energy transfer (BRET)-enhancing activity. Human embryonic kidney 293 cells co-expressing M_2 mAChR-Renilla luciferase and M_2 mAChR-yellow fluorescent protein were incubated for 60 min with 50 μM control immunoglobulin (Ig)G alone, 50 μM non-anti-pM_2 Chagas' disease (ChD) IgG alone, 50 μM ChD IgG or 2 μM anti-pM_2 ChD IgG supplemented with 48 μM of either control IgG or non-anti-pM_2 ChD IgG (a). Alternatively, the same cell preparation was treated with various concentrations of anti-pM_2 ChD IgG in the presence of supplementary amounts of non-anti-pM_2 ChD or control IgG, keeping a total IgG concentration of 50 μM in either case (b). ΔBRET values are presented as mean ± standard error of the mean of four to six different patients tested in three to five independent experiments. Significant differences *versus* control IgG or non-anti-pM_2 ChD IgG (***$P < 0{\cdot}001$).

100 μM gallamine (allosteric ligand) or 10 μM atropine (orthosteric ligand) for 60 min at room temperature. These treatments were carried out in the presence of low ionic strength buffer conditions (5 mM Na/KPO_4, BSA 0·1%, pH 7·4), which favour allosteric interactions [28]. Under these conditions, gallamine – but not atropine – inhibited the stimulatory effect of ChD IgG on BRET, whereas neither ligand was able to modify BRET values from membranes treated with buffer alone or control IgG (Fig. 5).

Together, these data indicate that the effects of anti-M_2 mAChR antibodies on BRET involve the recognition of the common allosteric site at the II-ECL of M_2 mAChR.

Role of antibody valency on BRET enhancing activity

Unlike the undigested ChD IgG fraction, its derived Fab fragment was unable to induce a significant increase in the BRET signal (Fig. 6). In fact, the BRET levels induced by ChD Fab were similar to those obtained in the presence of the Fab fragment derived from control IgG. However, the addition of goat anti-human Fab IgG rescued the ability of ChD Fab, but not control Fab, to enhance BRET. Simultaneous addition of isotype-control goat IgG together with either ChD or control Fab fragments did not lead to a significant recovery of the BRET signal. Taken together, these results suggest that the enhancement of M_2 receptor–receptor interaction induced by anti-M_2 mAChR antibodies occurs through cross-linking by bivalent antibodies.

Discussion

Given that BRET is a consequence of protein–protein interaction, and that RLuc and YFP do not interact with each other when expressed as non-fusion proteins, a significant BRET signal between M_2-RLuc and M_2-YFP (basal BRET ratio) has been interpreted previously as constitutive M_2 mAChR dimerization [17]. In the present study, a significant increase in the BRET ratio over the basal value was detected in the presence of the IgG fraction from ChD patients, suggesting that ChD antibodies interact with adjacent receptors,

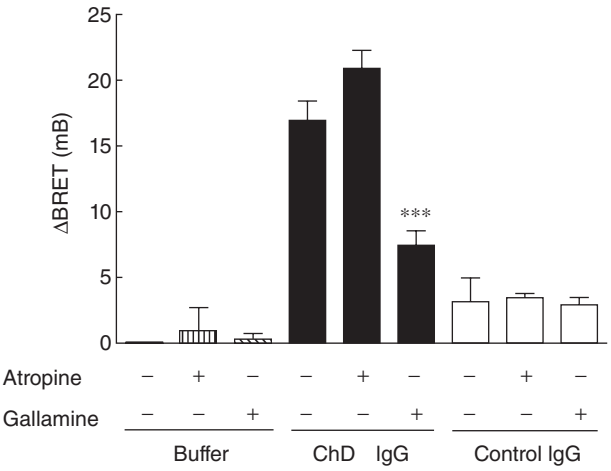

Fig. 5. Modulation by orthosteric and allosteric ligands of bioluminescence resonance energy transfer (BRET)-enhancing activity. A suspension of membranes from human embryonic kidney 293 cells co-expressing M_2 mAChR-Renilla luciferase and M_2 mAChR-yellow fluorescent protein in a low ionic strength buffer (5 mM Na/KPO_4/bovine serum albumin 0·1%, pH 7·4) was incubated for 60 min at room temperature with 50 μM control immunoglobulin (Ig)G or Chagas' disease (ChD) IgG in the presence or absence of 100 μM gallamine or 10 μM atropine. ΔBRET values are presented as mean ± standard error of the mean of three different patients performed in triplicate. Significant differences *versus* ChD IgG alone or ChD IgG + atropine (***$P < 0{\cdot}001$).

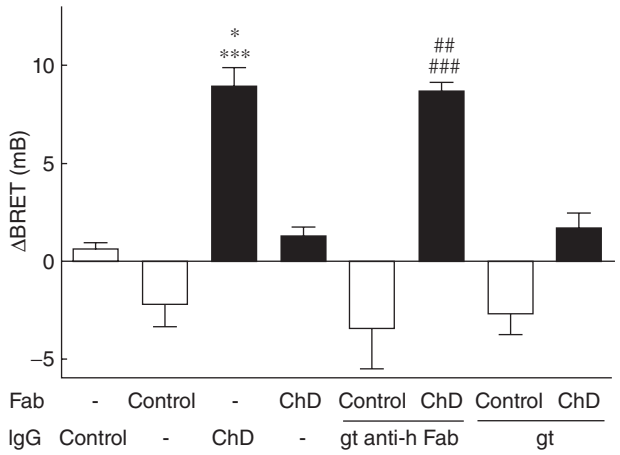

Fig. 6. Role of antibody valency in the modulation of M_2 muscarinic receptor–receptor interaction. Human embryonic kidney 293 cells co-expressing M_2 mAChR-Renilla luciferase and M_2 mAChR-yellow fluorescent protein were incubated for 60 min with Chagas' disease (ChD) or control Fab fragments (50 μM) in the presence or absence of goat anti-human Fab IgG (gt anti-h Fab IgG) or goat IgG (gt IgG). Alternatively, cells were treated with ChD IgG alone (25 μM) or control IgG alone (25 μM). Δ-bioluminescence resonance energy transfer (ΔBRET) values are presented as mean ± standard error of the mean of three to five different patients tested in three to five independent experiments. Significant differences: *versus* control IgG, ChD Fab or ChD Fab + gt IgG (*$P < 0{\cdot}05$); *versus* control Fab, control Fab + gt anti-h Fab IgG, control Fab + gt IgG (***$P < 0{\cdot}001$); *versus* ChD Fab + gt IgG (##$P < 0{\cdot}01$); *versus* control Fab + gt anti-h Fab IgG or control Fab + gt IgG (###$P < 0{\cdot}001$).

and subsequently enhance M_2 receptor–receptor interaction. The observed effect appears to be antibody- and receptor-specific. In fact, serum IgG from control subjects induced a minimum BRET signal, similar to basal BRET values. ChD IgG did not promote an increase in energy transfer between fusion proteins derived from a transmembrane receptor different from the M_2 mAChR. The fact that constitutive M_3 receptor–receptor interaction was not modified by ChD IgG antibodies was not surprising, as none of the ChD sera involved in this study showed positive immunoreactivity against the II-ECL of M_3 mAChR [29] by ELISA (data not shown), in agreement with our previous report [5]. In addition, exposure of cells co-expressing M_2-RLuc and M_3-YFP or their reverse combination to ChD IgG did not result in BRET enhancement. These results suggest that, even if ChD IgG antibodies promote an increase in the proximity between two M_2 receptors involved in preformed M_2–M_3 heterodimers, such an arrangement does not favour M_2–M_3 receptor–receptor interactions.

The BRET signal induced by ChD IgG on the M_2-RLuc/M_2-YFP cell system did not result simply from the ability of this antibody fraction to bind to or activate the M_2 mAChR in the manner of conventional muscarinic ligands. In fact, both orthosteric and allosteric muscarinic ligands failed to mimic the effect of anti-M_2 mAChR antibodies on our BRET system (Figs 3 and 5).

It could be argued that these antibodies promote a particular active conformation of the M_2 receptor – different from that induced by conventional muscarinic agonists – which could favour the interaction between transmembrane domains from adjacent receptors. However, addition of 10 μM atropine, which has been shown to block activation of M_2 mAChR by ChD antibodies [1–3,9–11], could not prevent the enhancing effect of ChD IgG on BRET in cells co-expressing M_2-RLuc and M_2-YFP, thus suggesting that this effect does not occur as a consequence of receptor activation.

Because ChD IgG antibodies can regulate the expression and function of M_2 mAChR by inducing receptor desensitization and sequestration [30], it could still be conceived that BRET enhancement is promoted by receptor endocytosis. First, pre-treatment of cells with atropine, which is supposed to block agonist-mediated M_2 mAChR endocytosis, did not impair the effect of ChD IgG on BRET. Secondly, ChD IgG also promoted BRET enhancement in membranes obtained from cells co-expressing M_2-RLuc and M_2-YFP, which indicates that the observed effect takes place at the membrane level. Therefore, the hypothesis that the effect of ChD IgG on BRET results from the recruitment of clustered receptors into endocytic vesicles seems unlikely.

In this study, two main differential properties between conventional agonists and agonist-like anti-G protein-coupled receptor (GPCR) antibodies were analysed: binding site on the receptor molecule and valency of ligand. While pharmacological agonists are monovalent and interact with receptor transmembrane residues, agonist-like antibodies are bivalent molecules that recognize extracellular receptor domains. We found that monospecific fractions of ChD IgG antibodies to the II-ECL (anti-pM_2 ChD IgG) of human M_2 AChR can promote an increase in BRET to the same extent as the total serum IgG from the same patients. Moreover, removal of anti-II-ECL antibodies from the whole ChD IgG fraction generated a non-anti-peptide fraction (non-anti-pM_2 ChD IgG), which failed to modulate M_2 receptor–receptor interaction. In addition, muscarinic allosteric modulator gallamine inhibited the ChD IgG-mediated increase in BRET under ionic strength conditions that favour allosteric interactions. Taken together, these findings suggest that the enhancement of M_2 receptor–receptor interaction by serum ChD IgG antibodies is mediated by the recognition of the common allosteric site at the receptor's II-ECL. This conclusion is supported by the fact that muscarinic ligands interacting with the orthosteric binding site of the M_2 receptor, such as carbachol and atropine, could not modify the effect of ChD IgG on BRET (Supplementary Fig. S1 and Fig. 5).

With regard to the role of the valency of ChD IgG antibodies on the modulation of M_2 receptor–receptor interaction, we conclude that this effect depends on the integrity of

the antibody molecules. Moreover, the ability of anti-Fab antibodies to restore BRET to a similar extent as the complete IgG antibody suggests that bivalent anti-M_2 mAChR IgG antibodies can bridge adjacent M_2 fusion proteins, thus bringing them into close proximity.

In a previous report using the same BRET methodology as we used in the present study, we provided evidence suggesting that the receptor population in HEK 293 cells only expressing the M_2 subtype is composed predominantly of constitutive homodimers (78%) [17]. More recently, FRET-based studies were used to estimate the size of oligomers formed by the M_2 mAChR, and concluded that this receptor subtype is most probably a tetramer [19]. In view of these findings, our data suggest that the enhancement in BRET promoted by ChD IgG is more probably a consequence of the clustering of constitutive preformed dimers or higher-order oligomers than the result of cross-linking of monomeric receptors. In fact, the relatively modest increase in BRET (20–25%) induced by ChD IgG antibodies supports the previous interpretation and also suggests that these antibodies could cross-link adjacent receptors involved in preformed dimers (or oligomers), thereby stabilizing pre-established receptor–receptor interactions.

Based on previous studies and the present results, we cannot yet define the pharmacological implications of the effect of ChD antibodies on M_2 mAChR receptor–receptor interaction. The basic role of homotropic M_2 receptor–receptor interaction in muscarinic receptor pharmacology still remains to be elucidated. However, previous data have provided some insight that deserves further discussion. Elies *et al*. showed that a monoclonal antibody against the II-ECL of human M_2 mAChR, but not its derived Fab fragment, promoted a negative inotropic effect on rat cardiomyocytes through the activation of M_2 mAChR [31]. Moreover, the agonist-like activity of this antibody was restored by cross-linking the monovalent fragments with anti-mouse IgG. Because bivalent ChD antibodies can both enhance M_2 receptor–receptor interaction and activate the receptor, we propose that such enhanced interaction could contribute to stabilize the active conformation.

Previous studies have provided evidence supporting the role of anti-M_2 mAChR antibodies in ChD pathophysiology. Muscarinic agonist-like activity of ChD IgG on the oesophageal smooth muscle was proposed to be involved in the predominantly excitatory unbalance characteristic of chagasic achalasia [4]. In addition, long-term exposure of M_2 mAChR from both myocardium [1,3,6,9–13,32] and colonic smooth muscle [5] to anti-M_2 mAChR antibodies is believed to have a causative role in parasympathetic dysfunction (dysautonomia) [1–3,33] and megacolon [5], respectively, probably via receptor desensitization and sequestration [30]. The present findings, together with those from Elies *et al*. [31], suggest that the enhanced M_2 receptor–receptor interaction induced by bivalent anti-M_2 mAChR IgG antibodies could favour M_2 mAChR activation, and consequently trigger the extensively described agonist-like effects that result ultimately in achalasia, dysautonomia and megacolon.

In conclusion, our data demonstrate that the serum IgG fraction from ChD patients can enhance M_2 receptor–receptor interaction, and suggest that this effect occurs as a result of receptor cross-linking by bivalent antibodies directed against the receptor's II-ECL. The present study provides a novel insight into the pathophysiological mechanism of anti-autonomic receptor antibodies. In addition, it proposes a sensitive methodology to detect anti-autonomic receptor antibodies in patients suffering from ChD and other pathologies involving such antibodies.

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Disclosure

None of the authors has any conflict of interests with the subject matter or materials discussed in this manuscript.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Effects of muscarinic ligands on bioluminescence resonance energy transfer (BRET)-enhancing activity promoted by Chagas' disease (ChD) antibodies. Human embryonic kidney 293 cells co-expressing M_2 mAChR-Renilla luciferase and M_2 mAChR-yellow fluorescent protein were incubated for 60 min with Krebs–Ringer–HEPES supplemented with 0.1% BSA (KRHA) buffer or 50 μM ChD immunoglobulin (Ig)G in the presence or absence of 10 μM atropine and/or 10 μM carbachol. ΔBRET values are presented as mean ± standard error of the mean of three to five different patients tested in three to five experiments.

Fig. S2. Assessment of immunoreactivity against the second extracellular loop (II-ECL) of the human M_2 muscarinic acetylcholine receptor of eluted immunoglobulin (Ig)G fractions after affinity chromatography by enzyme-linked immunosorbent assay. Muscarinic peptide pM_2 was coated onto microtitre wells, and incubated further with increasing concentrations of anti-pM_2 Chagas' disease (ChD) IgG (anti-pM_2) or non-anti-pM_2 ChD IgG (non-anti-pM_2),

which had been pre-incubated with or without 100 μM soluble pM_2. Optical density $(OD)_{405nm}$ values are mean ± standard error of the mean of five IgG fractions, performed in triplicate. Significant differences *versus* anti-pM_2 ChD IgG + pM_2, non-anti-pM_2 ChD IgG or non-anti-pM_2 ChD IgG + pM_2 (**$P < 0{\cdot}01$, ***$P < 0{\cdot}001$).

Table S1. Effect of Chagas' disease (ChD) immunoglobulin (Ig)G on M_2 muscarinic receptor–receptor interaction in cells. Specificity of the carrier protein.

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